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# Laboratory Diagnostic Systems for Ebola and Marburg Hemorrhagic Fevers Developed with Recombinant Proteins

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*Ebola virus* and *Marburg virus* (EBOV and MARV, respectively) of the family *Filoviridae* cause hemorrhagic fever with high mortality rates, sometimes reaching 50 to 90% of infected individuals, in humans and nonhuman primates (10, 16, 47). EBOV consists of four species, Zaire EBOV, Sudan EBOV, Ivory Coast EBOV, and Reston EBOV, which were first isolated in the Democratic Republic of Congo, Sudan, Ivory Coast, and the Philippines, respectively (47). MARV consists of one species, Lake Victoria MARV. Public health concerns about filovirus infection have increased in recent years. First, there have recently been large outbreaks of hemorrhagic fevers caused by EBOV (Ebola hemorrhagic fever [EHF]) and MARV (Marburg hemorrhagic fever [MHF]) in Africa; EHF outbreaks occurred in the Democratic Republic of Congo and Uganda in 1995 and 2000, respectively, and MHF outbreaks occurred in the Democratic Republic of Congo in 1998–1999 and in Angola in 2004–2005 (1, 7, 27, 56, 58–60). Second, there is a possibility that filoviruses may be used as bioweapons. In this regard, filoviruses are classified as category A warfare agents by the U.S. government and are considered to pose a great risk to international security, along with anthrax, botulism, tularemia, and smallpox (2).

As the magnitude of international trade and travel is continuously increasing, there is a significant risk that the hemorrhagic fever viruses could be introduced to virus-free countries from areas where they are endemic. Therefore, the development of laboratory diagnostic systems for EHF and MHF is an important subject even in countries without viral hemorrhagic fevers. Manipulation of infectious hemorrhagic fever viruses such as EBOV, MARV, Crimean-Congo hemorrhagic fever virus, and Lassa virus requires a biosafety level 4 (BSL-4) laboratory, which is designed for work with dangerous and exotic agents that pose a high risk of laboratory infection and life-threatening disease. However, BSL-4 laboratories are only available in a limited group of countries, such as the United States, Canada, France, the United Kingdom, Germany, South Africa, Sweden, and Russia.

To get around the need for BSL-4 laboratories, recombinant viral antigens are used for immunodiagnostics. The recombinant proteins of these viruses have been expressed, and serological diagnostic methods have been developed using the recombinant proteins. Antigen detection systems have also been developed using recombinant antigens. In this article, recent progress in the development of diagnostic methods for EHF and MHF is reviewed.

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## EBOLA AND MARBURG HEMORRHAGIC FEVERS

**Structure of EBOV and MARV virions.** Electron microscopic examination revealed that EBOV and MARV virions are pleomorphic, appearing as either long filamentous forms or in shorter U-shaped, 6-shaped, or circular configurations. The filamentous forms vary greatly in length (up to 14,000 nm), with mean unit lengths of virions of about 1,200 and 860 nm for EBOV and MARV, respectively (47). The virus genome of EBOV is almost 19 kb long and encodes seven viral proteins, namely, nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein (GP), replication-transcription protein (VP30), matrix protein (VP24), and RNA-dependent RNA polymerase (L), with an additional soluble glycoprotein (sGP) produced from an edited GP mRNA. The genes are arranged in the order 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′. The virus genome of MARV has similar characteristics to EBOV, except for the expression of the soluble glycoprotein produced from edited GP mRNA (47). The nucleocapsid complexes of filoviruses consist of the nonsegmented negative-strand RNA genome, NP, polymerase L, VP35, and VP30. The structural proteins, VP40 and VP24, represent viral matrix proteins connecting the nucleocapsid to the viral envelope. The envelope GP is an integral membrane protein which forms spike-like protrusions on the surface of the virion (11, 13). GP mediates virus entry into susceptible cells through receptor binding and plays an important role in inducing neutralizing antibodies.

**Diseases caused by EBOV and MARV.** Filovirus infections, in general, are the most severe of the viral hemorrhagic fevers. Humans are usually infected with EBOV or MARV through close contact with the contaminated blood, tissues, and/or excretions of viremic animals, including patients with filovirus infections. After an incubation period of 4 to 10 days, infected individuals abruptly develop flu-like symptoms characterized by fever, chills, malaise, and myalgia. Subsequently, patients usually develop the signs and symptoms that indicate systemic involvement, such as prostration and gastrointestinal (anorexia, nausea, vomiting, abdominal pain, and diarrhea), respiratory (chest pain, shortness of breath, and cough), vascular (conjunctival injection, postural hypotension, and edema), and neurological (headache, confusion, and coma) manifestations.

TABLE 1. Outbreaks of EHF and MHF

| Virus | Country(ies) | Year(s) | No. of deaths/no. of patients | Description |
|---|---|---|---|---|
| Lake Victoria MARV | Germany and Serbia/Herzegovina | 1967 | 7/31 | Origin of MARV responsible for the outbreak was a monkey imported from Uganda. |
| | Zimbabwe and South Africa | 1975 | 1/3 | Index case was infected with MARV in Zimbabwe; nosocomial infection occurred in a hospital in South Africa. |
| | Kenya | 1980 | 1/2 | |
| | Kenya | 1987 | 1/1 | |
| | Democratic Republic of Congo | 1998–1999 | 52/76 | Prospective study on MHF indicated that there were approximately 150 patients with MHF in this outbreak. |
| | Angola | 2004–2005 | 357/423 (as of 7 July 2005) | Mortality rate exceeded 80%; many children suffered from MHF in this outbreak. |
| Sudan EBOV | Sudan | 1976 | 151/284 | First documented outbreak of VHF due to Sudan EBOV. |
| Zaire EBOV | Democratic Republic of Congo | 1976 | 280/318 | First documented outbreak of VHF due to Zaire EBOV. |
| Zaire EBOV | Democratic Republic of Congo | 1977 | 1/1 | Sporadic outbreak. |
| Sudan EBOV | Sudan | 1979 | 22/34 | |
| Reston EBOV | United States | 1989–1990 | 0/4 | Outbreak of EHF in nonhuman primates imported from the Philippines. Four persons were confirmed to be infected with Reston EBOV without any symptoms. |
| Reston EBOV | Italy | 1992 | 0/0 | Outbreak of EHF in nonhuman primates imported from the Philippines. |
| Ivory Coast EBOV | Ivory Coast | 1994 | 0/1 | A veterinarian was infected when she handled a dead chimpanzee. |
| Zaire EBOV | Democratic Republic of Congo | 1995 | 244/315 | The epicenter of this outbreak is Kikwit, Democratic Republic of Congo. |
| Zaire EBOV | Gabon | 1996 | 21/31 | |
| Zaire EBOV | Gabon and South Africa | 1996 | 45/60 | Nosocomial infection occurred in a hospital in which a nurse who took care of a doctor that was transferred from Gabon died. |
| Sudan EBOV | Uganda | 2000 | 149/394 | The epicenter of this EHF outbreak was the Gulu District in Uganda. |
| Zaire EBOV | Gabon and the Democratic Republic of Congo | 2001–2002 | 69/92 | The outbreak area was close to the border between the two countries. |
| Zaire EBOV | Democratic Republic of Congo | 2003 | 29/35 | Thirteen of the cases were laboratory confirmed and 130 were epidemiologically linked. |
| Sudan EBOV | Sudan | 2004 | 7/17 | |
| Zaire EBOV | Democratic Republic of Congo | 2005 | 9/12 (as of 16 June 2005) | One case was virologically confirmed (http://www.who.int/csr/don/2005_06_16/en/index.html). |

Bleeding is manifested as petechiae, ecchymosis, uncontrolled oozing from venipuncture sites and gingiva, mucosal hemorrhages, and bloody diarrhea. In later stages, the general condition of patients deteriorates due to multiorgan failure, including disseminated intravascular coagulopathy, resulting in death (4, 14, 42, 47).

**Epidemiology of Ebola and Marburg hemorrhagic fevers.** The outbreaks caused by EBOV and MARV are summarized in Table 1. The first documented MHF outbreak occurred in Germany and then in Yugoslavia in 1967 (36). Technicians and scientists suffered from MHF after they manipulated tissue materials collected from African green monkeys imported from Uganda. It was suggested that the monkeys had already been infected with MARV when imported. Three sporadic cases of MHF were reported in Zimbabwe (1975) and Kenya (1980 and 1987) (8, 12, 24, 49). From 1998 to 1999, there was a large outbreak in the Democratic Republic of Congo (1). The largest outbreak of MHF occurred in Uige Province, Angola, in 2004, and 374 patients have been reported, with a mortality rate of over 88%, as of 24 August 2005 (http://www.who.int/csr/don/2005_08_24/en/index.html).

The first recognized outbreaks of EHF occurred in the Democratic Republic of Congo, formerly Zaire, and Sudan in 1976 (3, 26, 54, 55). After the discovery of EBOV in 1976, several African countries were struck by outbreaks of EHF caused by one of the three known human-pathogenic EBOV species (Zaire EBOV, Sudan EBOV, and Ivory Coast EBOV) (12, 17, 27, 31, 52, 57–59, 61, 62). Outbreaks of EHF caused by the other EBOV strain, Reston EBOV, occurred among cynomolgus macaques imported from the Philippines to the United States in 1989 (23). Reston EBOV was also introduced to the United States in 1989, 1990, and 1996, and to Italy in 1992, through importation of infected monkeys from the Philippines (5, 6, 23, 63). It is noteworthy that the incidences of EHF and MHF outbreaks are increasing and that the number of patients suffering from EHF or MHF is on the rise (Table 1).

Several cases of nosocomial infections of EBOV and MARV outside the areas of endemicity have been reported. A health-

care worker who took care of severely ill patients transported from Zimbabwe acquired MARV infection (15), and a health-care worker was infected with Zaire EBOV after taking care of a severely ill patient transported from Gabon (41). The severely ill patient was a medical doctor who was infected with Zaire EBOV from an EHF patient in Gabon. Both nosocomial infections occurred in South Africa.

## CURRENT LABORATORY DIAGNOSTICS FOR EHF AND MHF

**Overview of current laboratory diagnostics for EHF and MHF.** In outbreaks of EHF and MHF, infections are confirmed by various laboratory diagnostic methods. These include virus isolation, reverse transcription-PCR (RT-PCR), including real-time quantitative RT-PCR, antigen-capture enzyme-linked immunosorbent assay (ELISA), antigen detection by immunostaining, and IgG- and IgM-ELISA using authentic virus antigens (9, 18, 28–30, 32, 48, 50, 53, 64). Histological techniques, including antigen detection by immunohistochemical analyses, are sensitive methods, particularly for postmortem diagnosis (64). Diagnosis by detection of virus antigens is suitable for patients in the early stage of illness, while serological diagnosis by the detection of specific IgM and IgG antibodies is suitable for patients in a relatively late stage of illness. The former is especially suitable for patients who die before an antibody response is mounted. Diagnostics for viral hemorrhagic fevers, including EHF and MHF, must be sensitive, specific, and reliable because misdiagnosis of viral hemorrhagic fevers may bring huge turmoil to society. Therefore, the diagnosis of EHF and MHF must not rely on any single diagnostic method alone. The risk of misdiagnosis must be extremely minimized. In actual EHF or MHF outbreak areas, patients with EHF or MHF must be isolated. This indicates that a false-positive result will put an individual at unnecessary risk of infection by making the person be placed in a high-risk environment such as an isolation ward. A false-negative result will allow persons who are infected with EBOV or MARV to be released into the community with the understanding that they do not have viral hemorrhagic fever, when in fact they have the potential to become highly contagious and cause person-to-person transmission of these viruses in the community. In Africa, Lassa fever and Crimean-Congo hemorrhagic fever are also endemic. Therefore, the diagnosis of viral hemorrhagic fevers must rely on multiple diagnostic assays for viral hemorrhagic fevers in a comprehensive manner.

**Virus isolation.** Virus isolation is a basic, simple, and sensitive method for diagnosis of EHF and MHF. EBOV and MARV grow well in a large variety of cell lines, but Vero cells and Vero E6 cells are commonly used. Specimens such as blood must be sent to BSL-4 laboratories, which are located in developed countries, from an outbreak area, which is often very remote. The shipment of specimens for virus isolation under favorable conditions (cold chain during the period from shipment to arrival) is often difficult. Therefore, diagnostic criteria based on virus isolation alone will not yield an etiologic diagnosis. A panel of monoclonal antibodies to the recombinant NPs (rNPs) of Zaire EBOV, Reston EBOV, and Lake Victoria MARV was established by our group (20, 39, 40, 44). Monoclonal antibodies specific only for NP of Zaire EBOV, for NP of Reston EBOV, for NPs of Zaire and Sudan EBOV, for NPs of Zaire and Reston EBOV, and for NP of Lake Victoria MARV alone were developed. By using these monoclonal antibodies, it became possible to serologically identify the species of EBOV and MARV isolates.

**RT-PCR, including real-time quantitative RT-PCR.** Molecular diagnostic methods for EHF and MHF by RT-PCR were developed and evaluated well in the epidemic setting, especially in the EHF outbreaks in the Democratic Republic of Congo in 1995, in Gabon in 1996, and in Uganda in 2000 (32, 48, 50). During the EHF outbreak in Uganda in 2000, a nested RT-PCR using primer sets designed specifically for the NP region of the Sudan EBOV Gulu strain, which was the causative EBOV for the outbreak, was developed (50). Recently, real-time quantitative RT-PCR methods for EHF and MHF were also developed (9, 18, 50, 53). The real-time quantitative RT-PCR method developed by Drosten et al. is a one-step RT-PCR with the addition of the DNA-intercalating dye SYBR green I using the primer set Filo-A (5′-ATCGGAATT TTTCTTTCTCATT-3′) and Filo-B (5′-ATGTGGTGGGTTA TAATAATCACTGACATG-3′), which was designed for the amplification of L genes from both EBOV and MARV (Table 2) (9, 48). On the other hand, the real-time quantitative RT-PCR methods developed by other investigators are based on the technology of TaqMan probe-based quantitative RT-PCR, using a designated primer set with a fluorogenic TaqMan probe that is labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and at the 3′ end with a quencher tag (18, 50, 53). The primer sequences used for RT-PCR, nested RT-PCR, and real-time quantitative RT-PCR are summarized in Table 2. These molecular diagnostics for EHF and MHF have been proven to be sensitive, specific, and efficacious in the diagnosis of filovirus infections. Although RT-PCR assays, especially nested RT-PCR and real-time quantitative RT-PCR, are useful, false-positive and false-negative results must always be excluded. The sensitivities of the RT-PCR systems used in several laboratories vary (38).

**Antigen detection ELISAs for EHF and MHF.** When infection with EBOV or MARV becomes fatal, patients usually die before the antibody response. This fact suggests that serological diagnostics are suitable for the diagnosis of infection in patients who survive but not in those who succumb to the infection. High titers of infectious filovirus are present in the blood and tissues of patients at the early stage of illness, suggesting that the detection of virus antigens is suitable for diagnosis of EHF and MHF at an early stage. Antigen-capture ELISA was developed for the detection of EBOV antigens; it was used in clinical settings such as EHF outbreaks in the Democratic Republic of Congo in 1995, in Gabon in 1996, and in Uganda in 2000 and was confirmed to be efficacious in diagnosis of EHF (28–30, 50). In the antigen-capture ELISA, a pool of monoclonal antibodies to the Zaire and Sudan EBOVs and rabbit sera raised to the Zaire and Sudan EBOVs were used as capture and detection antibodies, respectively (28, 29). Antigen-capture ELISA systems to detect EBOV and MARV antigens have also been developed by other groups, including ours (20, 34, 35, 39, 44). The target proteins are NP, VP40, and GP. The characteristics of the developed EBOV- and MARV-specific antigen-capture ELISAs are summarized in Table 3. Saijo et al. developed filovirus antigen detection

TABLE 2. Primers used for RT-PCR amplification of EBOV sequences and targeted genes, with predicted sizes of amplified DNA products

| Assay system | Primer or probe[a] | Sequence (sense) (5′–3′) | Target gene (size of amplicon [bp]) | Reference |
|---|---|---|---|---|
| RT-PCR | EBO-GP1 (F) | AATGGGCTGAAAATTGCTACAATC | EBOV GP (579) | 48 |
| | EBO-GP2 (R) | TTTTTTTAGTTTCCCAGAAGGCCCACT | | |
| RT-PCR | FILO-A (F) | ATCGGAATTTTTCTTTCTCATT | Filovirus L (419) | 48 |
| | FILO-B (R) | ATGTGGTGGGTTATAATAATCACTGACATG | | |
| RT-PCR | RES-NP1 | GTATTTGGAAGGTCATGGATTC | Reston EBOV NP (337) | 48 |
| | RES-NP2 (R) | CAAGAAATTAGTCCTCATCAATC | | |
| RT-PCR | ZAI-NP1 | GGACCGCCAAGGTAAAAAATGA | Zaire EBOV NP (268) | 48 |
| | ZAI-NP2 (R) | GCATATTGTTGGAGTTGCTTCTCAGC | | |
| Nested RT-PCR[c] | SudZaiNP1 (+) (F) | GAGACAACGGAAGCTAATGC | Sudan and Zaire EBOV NP (150) | 50 |
| | SudZaiNP1 (−) (R) | AACGGAAGATCACCATCATG | | |
| | SudZaiNP2 (+) (F) | GGTCAGTTTCTATCCTTTGC | | |
| | SudZaiNP2 (−) (R) | CATGTGTCCAACTGATTGCC | | |
| Real-time qRT-PCR[b] | Unnamed (F) | GAAAGAGCGGCTGGCCAAA | NP ORF region of the Gulu strain of Sudan EBOV (78) | 50 |
| | Unnamed (R) | AACGATCTCCAACCTTGATCTTT | | |
| | Unnamed (P) | TGACCGAAGCCATCACGACTGCAT | | |
| Real-time qRT-PCR[b] | EBOGP-1D forward (F) | TGGGCTGAAAAYTGCTACAATC | GP region of Zaire EBOV (112) | 18 |
| | EBOGP-1D reverse (R) | CTTTGTGMACATASCGGCAC | | |
| | EBOGP-1DZPrb (P) | CTACCAGCAGCGCCAGACGG | | |
| Real-time qRT-PCR[b] | EBOGP-1D forward (F) | TGGGCTGAAAAYTGCTACAATC | GP region of Sudan EBOV, Boniface strain (112) | 18 |
| | EBOGP-1D reverse (R) | CTTTGTGMACATASCGGCAC | | |
| | EBOGP-1DSPrb (P) | TTACCCCCACCGCCGGATG | | |
| Real-time qRT-PCR[b] | ENZ FP (F) | ATGATGGAAGCTACGGCG | NP region of Zaire EBOV (70) | 53 |
| | ENZ RP (R) | AGGACCAAGTCATCTGGTGC | | |
| | ENZ P (P) | CCAGAGTTACTCGGAAAACGGCATG | | |
| Real-time qRT-PCR[b] | ENS FP (F) | TTGACCCGTATGATGATGAGAGTA | NP region of Sudan EBOV, Boniface strain (88) | 53 |
| | ENS RP (R) | CAAATTGAAGAGATCAAGATCTCCT | | |
| | ENS P (P) | CCTGACTACGAGGATTCGGCTGAAGG | | |
| Real-time qRT-PCR[b] | MN FP (F) | CAATTCCACCTTCAGAAAACTG | NP region of LV MARV,[d] Popp strain (77) | 53 |
| | MN RP (R) | GCTAATTTTTCTCGTTTCTGGCT | | |
| | MN P (P) | CACACACAGTCAGACACTAGCCGTCCT | | |

[a] Primers followed by (F), (R), and (P) indicate forward, reverse, and probe primers, respectively.
[b] qRT-PCR, quantitative RT-PCR.
[c] The primers SudZaiNP1 (+) and SudZaiNP1 (−) are used for the first-round PCR reaction, and the primers SudZaiNP2 (+) and SudZaiNP2 (−) are used for the second-round PCR reaction. The size of the PCR products of 150 bp is expected when the amplicon is generated with the primer set designed for the second-round PCR.
[d] LV MARV, Lake Victoria MARV.

ELISAs using unique monoclonal antibodies to the rNPs of Zaire EBOV, Reston EBOV, and Lake Victoria MARV (20, 39, 44). Although the monoclonal antibodies were produced by immunizing mice with recombinant NPs, the NP-capture ELISA detected not only the rNPs of these viruses but also the authentic EBOV and MARV rNPs. Antigen-capture ELISAs were developed for detecting the NPs of Zaire EBOV, Sudan EBOV, and Reston EBOV (39), that of Reston EBOV alone (20), and that of MARV alone (44). The antigenic regions on the NPs of EBOV and MARV were determined to be located in their carboxy-terminal halves. The carboxy-terminal 110 and 102 amino acids of the NPs of EBOV and MARV, respectively, possess strong antigenicity (45). All the monoclonal antibodies that could be used as capture antibodies in the antigen-capture ELISA format reacted with epitopes within the carboxy-terminal ends of NPs (20, 39, 44). The monoclonal antibodies useful as capture antibodies for rNPs of EBOV and MARV recognized conformational epitopes within the carboxy-terminal ends of NPs, while those for rNP of Reston EBOV recognized linear epitopes (20, 39, 44). Interestingly, the antigen-capture ELISA with capture monoclonal antibodies to the rNP of Reston EBOV (Res2-6C8 and Res2-1D8), which recognize linear epitopes, can detect only rNP of Reston EBOV, but that with capture antibody to rNP of Zaire EBOV (3-3D), which recognizes the conformational epitope, can detect not only the rNP of Zaire EBOV but also rNPs of Sudan, Reston, and Ivory Coast EBOVs (20, 39). Lucht et al. developed an antigen-capture ELISA using a monoclonal antibody to Zaire EBOV GP as the capture antibody and another monoclonal antibody to the same antigen as a detector antibody (35). They also developed an ELISA for the detection of Zaire EBOV VP40, using two monoclonal antibodies to Zaire EBOV VP40 as capture and detector antibodies (34). The EBOV GP-capture ELISA only detected the GP of Zaire EBOV, and the EBOV VP40-capture ELISA detected the VP40s of all four EBOV species. It is considered that EBOV and MARV antigen detection ELISAs are useful for accurate and rapid diagnosis of EHF and MHF. The efficacies of these antigen-capture ELISAs must be evaluated by using patient specimens in a clinical setting and in actual EHF or MHF outbreaks.

**Diagnosis by detection of antibodies to EBOV and MARV, including recombinant protein-based antibody detection systems.** An indirect immunofluorescence assay developed with authentic virus antigens made from virus-infected cells has been commonly used as a tool for the detection of antibodies to filoviruses (25, 51). It has shown a high sensitivity for antibodies during early convalescence and has been used exten-

TABLE 3. Characteristics of reported antigen-capture ELISAs for filovirus antigens, such as target proteins, capture and detector antibodies, and species of EBOV and MARV that react with each capture antibody

| Target protein | Capture antibody | Recognition site of capture antibody | Detector antibody | Reactivity[a] | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|
| | | | | EBOV | | | | LV MARV[b] | |
| | | | | Zaire | Sudan | Ivory Coast | Reston | | |
| EBOV | Pool of MAbs to Zaire and Sudan EBOVs | Unspecified | Rabbit sera raised to Zaire and Sudan EBOVs | R | R | UK | R | N | 28 |
| Zaire EBOV NP | MAb 3-3D to Zaire EBOV | Carboxy-terminal region of Zaire EBOV NP | Rabbit serum raised to Zaire EBOV rNP | R | R | PR | R | N | 39 |
| Reston EBOV NP | MAb Res2-6C8 to Reston EBOV NP | Carboxy-terminal region of Reston EBOV NP | Rabbit serum raised to Reston EBOV rNP | N | N | N | R | N | 20 |
| | MAb Res2-1D8 to Reston EBOV NP | Carboxy-terminal region of Reston EBOV NP | Rabbit serum raised to Reston EBOV rNP | N | N | N | R | N | 20 |
| Zaire EBOV GP | MAb 3B11 to Zaire EBOV GP | Unspecified | POD-labeled MAb 1G12 to Zaire EBOV GP | R | N | N | N | N | 35 |
| Zaire EBOV VP40 | MAb 2C4 to Zaire EBOV VP40 | Unspecified | Biotin-labeled MAb 5F6 to Zaire EBOV VP40 | R | R | R | R | N | 34 |
| LV MARV[b] NP | MAb 2A7 to MARV NP | Carboxy-terminal region of MARV NP (amino acid residues 632 to 645) | Rabbit serum raised to MARV rNP | N | N | N | N | R | 44 |
| | MAb 2H6 to MARV NP | Carboxy-terminal region of MARV NP (amino acid residues 643 to 695) | | N | N | N | N | R | |

[a] R, UK, N, and PR indicate reactive, unknown, nonreactive, and possibly reactive, respectively.
[b] LV MARV, Lake Victoria MARV.

sively in seroepidemiological surveys of EBOV and MARV antibody prevalence. Recently, an authentic EBOV antigen-based IgG-capture ELISA and IgM-capture ELISA were developed and used as tools for serological diagnosis of EBOV infections (29, 30, 50). The IgM-capture ELISA has great usefulness as a diagnostic tool for EHF. Furthermore, IgG-ELISA is efficacious, not only in diagnosis but also in field investigations of EBOV infections.

It is impossible, however, for institutes without BSL-4 facilities to prepare authentic filovirus antigens from the infectious viruses. Considering the increase in outbreaks of EHF and MHF, the increase in travelers, and the possibility of bioterrorism, diagnostic systems for viral hemorrhagic fevers are necessary even in countries where BSL-4 laboratories are not available. In order to overcome this difficulty, recombinant protein-based diagnostic systems for viral hemorrhagic fevers have been developed by several groups, including ours.

The EBOV and MARV antibody detection systems, in which recombinant proteins of EBOV and MARV are used as antigens, are summarized in Table 4. Prehaud et al. first reported the usefulness of the recombinant nucleoprotein (rNP) and glycoprotein (rGP) of Zaire EBOV strain Gabon 94 for the detection of IgG and IgM antibodies (43), and they studied the efficacy of antibody detection ELISAs developed with EBOV rNP expressed in an *Escherichia coli* system or EBOV rGP expressed in a baculovirus system. All sera collected from seven EHF patients showed positive reactions in both recombinant protein-based antibody-detection ELISAs. Twenty-two control sera showed negative reactions. Thus, the recombinant proteins were confirmed to be useful as antigens for detecting specific antibodies in IgG- and IgM-ELISAs. Saijo et al. further expressed rNPs of Zaire EBOV and MARV (Musoke strain) with a six-His tag at the amino-terminal end, using a baculovirus system (45). The rNP of Zaire EBOV was efficiently expressed. The Zaire EBOV rNP-based IgG-ELISA demonstrated a high sensitivity and specificity, not only for the detection of Zaire EBOV antibodies but also for the detection of Sudan EBOV and Reston EBOV antibodies (45). The NP of MARV, composed of 695 amino acid residues, and the carboxy-terminal half (341 to 695 amino acid residues) possessed strong antigenicity. The carboxy-terminal half of MARV rNP was expressed in an *E. coli* system with a glutathione *S*-transferase tag at the amino-terminal end. IgG-ELISA with the carboxy-terminal half of MARV-rNP was then developed. The IgG-ELISA with the carboxy-terminal half of MARV-rNP also demonstrated a high efficacy and specificity in detecting MARV antibodies (45). The results suggest that recombinant EBOV and MARV rNP-based serological diagnostics are useful for the diagnosis of and seroepidemiological investigations on viral hemorrhagic fevers. Using the same strategy, a Reston EBOV rNP-based IgG-ELISA system was developed (22). EBOV recombinant protein-based IgG-ELISA systems were also developed by other investigators. Groen et al. developed IgG-ELISAs using rNP or recombinant VP35 as antigens. It was demonstrated that a Zaire EBOV rNP-based ELISA detected IgG antibodies, not only to Zaire EBOV but also to other EBOV species, while a Zaire EBOV recombinant VP35 (rVP35)-based ELISA detected IgG antibodies to Zaire EBOV but not to other species. Furthermore, EBOV rNP-based IgG-ELISA was more sensitive in the detection of EBOV antibodies than was EBOV rVP35-based IgG-ELISA. It is noteworthy that the sensitivities and specificities of EBOV recombinant protein-based IgG-ELISAs were evaluated using a large panel of serum samples (26 positive

TABLE 4. Characteristics of recombinant antigen-based antibody detection systems

| Method | Origin of antigen | Antigen (amino acid positions)[a] | Expression of recombinant protein | Sensitivity (no. of positive samples/ no. of positive controls) | Specificity (no. of negative samples/ no. of negative controls) | Reference |
|---|---|---|---|---|---|---|
| ELISA | Zaire EBOV (Gabon 94 strain) | rNP | Transformation of *E. coli* with expression vector | 9/9 | 22/22 | 43 |
| | | rGP | Recombinant baculovirus system | 9/9 | 22/22 | |
| | Zaire EBOV | rNP | Recombinant baculovirus system | 13/14 | 50/51 | 45 |
| | | Truncated rNP (361–739) | Transformation of *E. coli* with expression vector | 13/14 | 50/51 | |
| | Zaire EBOV | rNP | Recombinant baculovirus system | 24/26 | 489/500 | 19 |
| | Zaire EBOV | rVP35 | Recombinant baculovirus system | 12/26 | 499/500 | 19 |
| | Reston EBOV | Truncated rNP (360–739) | Transformation of *E. coli* with expression vector | 10/10 | 72/72 | 22 |
| | LV MARV[b] | Truncated rNP (341–695) | Transformation of *E. coli* with expression vector | 3/3 | 62/62 | 45 |
| Indirect immunofluorescence assay | Zaire EBOV | rNP | Infection of HeLa cells with recombinant baculovirus | 14/14 | 47/48 | 46 |
| | Reston EBOV | rNP | Transfection of HeLa cells with expression vector | 16/16 | 96/96 | 21 |

[a] The amino acid positions are counted from the translational initiation codon for each protein.
[b] LV MARV, Lake Victoria MARV.

controls and 500 negative controls) collected from humans and nonhuman primates (19).

The immunofluorescence technique is an alternative method for detecting specific antibodies. Zaire EBOV rNP was expressed in HeLa cells by abortive infection with a recombinant baculovirus that carries the cytomegalovirus immediate-early promoter and the Zaire EBOV NP gene (46). Reston EBOV rNP was also expressed in HeLa cells by transfection with an expression vector, pKS336, carrying the Reston EBOV NP gene (21). The immunofluorescence systems developed with these EBOV rNP-expressing HeLa cells as antigens were confirmed to be highly sensitive and specific (21, 46).

A novel and unique antibody-phage indicator assay for detecting antibodies to the four species of EBOV in any species of animals was also developed (37). A human monoclonal antibody Fab fragment that reacts with an immunodominant epitope on EBOV NP that is conserved in all four EBOV species was established, and a competitive ELISA for detecting antibodies to EBOV was then developed using the Fab fragment. An assay using a phage-antibody spot test, which was able to detect antibodies to EBOV NP in the tested sera by inhibition of the reaction of the Fab antibody to EBOV antigens, was also developed. These assays are considered useful for the diagnosis of EBOV infections and for seroepidemiological studies in the field. Although some species of fruit bats were identified as reservoirs of EBOV (33), the greatest advantage is that this assay can be applied to seroepidemiological research of EBOV infections in a variety of animal species.

## CONCLUSIONS

New diagnostic systems for EHF and MHF have been developed recently, using EBOV and MARV recombinant proteins. These include IgG- and IgM-ELISAs, immunofluorescence techniques, and antigen-capture ELISAs with monoclonal antibodies induced by recombinant proteins. These diagnostic systems demonstrate high sensitivities and specificities and therefore are useful for the diagnosis of and epidemiological studies on filovirus infections. Furthermore, preparation of the antigens for these diagnostic systems does not require infectious filoviruses. Thus, the systems are especially advantageous for use in countries where BSL-4 facilities are not available.

## REFERENCES


1. **Bausch, D. G., M. Borchert, T. Grein, C. Roth, R. Swanepoel, M. L. Libande, A. Talarmin, E. Bertherat, J. J. Muyembe-Tamfum, B. Tugume, R. Colebunders, K. M. Konde, P. Pirad, L. L. Olinda, G. R. Rodier, P. Campbell, O. Tomori, T. G. Ksiazek, and P. E. Rollin.** 2003. Risk factors for Marburg hemorrhagic fever, Democratic Republic of the Congo. Emerg. Infect. Dis. **9:**1531–1537.
2. **Borio, L., T. Inglesby, C. J. Peters, A. L. Schmaljohn, J. M. Hughes, P. B. Jahrling, T. Ksiazek, K. M. Johnson, A. Meyerhoff, T. O'Toole, M. S. Ascher, J. Bartlett, J. G. Breman, E. M. Eitzen, Jr., M. Hamburg, J. Hauer, D. A. Henderson, R. T. Johnson, G. Kwik, M. Layton, S. Lillibridge, G. J. Nabel, M. T. Osterholm, T. M. Perl, P. Russell, and K. Tonat.** 2002. Hemorrhagic fever viruses as biological weapons: medical and public health management. JAMA **287:**2391–2405.
3. **Bowen, E. T., G. S. Platt, D. I. Simpson, L. B. McArdell, and R. T. Raymond.** 1978. Ebola haemorrhagic fever: experimental infection of monkeys. Trans. R. Soc. Trop. Med. Hyg. **72:**188–191.
4. **Bwaka, M. A., M. J. Bonnet, P. Calain, R. Colebunders, A. De Roo, Y. Guimard, K. R. Katwiki, K. Kibadi, M. A. Kipasa, K. J. Kuvula, B. B. Mapanda, M. Massamba, K. D. Mupapa, J. J. Muyembe-Tamfum, E. Ndaberey, C. J. Peters, P. E. Rollin, E. Van den Enden, and E. Van den Enden.** 1999. Ebola hemorrhagic fever in Kikwit, Democratic Republic of the Congo: clinical observations in 103 patients. J. Infect. Dis. **179**(Suppl. 1)**:**S1–S7.
5. **Centers for Disease Control and Prevention.** 1989. Ebola virus infection in imported primates—Virginia, 1989. Morb. Mortal. Wkly. Rep. **38:**831–832, 837–838.
6. **Centers for Disease Control and Prevention.** 1996. Ebola-Reston virus infection among quarantined nonhuman primates—Texas, 1996. Morb. Mortal. Wkly. Rep. **45:**314–316.

7. **Centers for Disease Control and Prevention.** 2001. Outbreak of Ebola hemorrhagic fever—Uganda, August 2000-January 2001. JAMA **285:**1010–1012.
8. **Conrad, J. L., M. Isaacson, E. B. Smith, H. Wulff, M. Crees, P. Geldenhuys, and J. Johnston.** 1978. Epidemiologic investigation of Marburg virus disease, Southern Africa, 1975. Am. J. Trop. Med. Hyg. **27:**1210–1215.
9. **Drosten, C., S. Gottig, S. Schilling, M. Asper, M. Panning, H. Schmitz, and S. Gunther.** 2002. Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J. Clin. Microbiol. **40:**2323–2330.
10. **Feldmann, H., S. Jones, H. D. Klenk, and H. J. Schnittler.** 2003. Ebola virus: from discovery to vaccine. Nat. Rev. Immunol. **3:**677–685.
11. **Feldmann, H., and M. P. Kiley.** 1999. Classification, structure, and replication of filoviruses. Curr. Top. Microbiol. Immunol. **235:**1–21.
12. **Feldmann, H., W. Slenczka, and H. D. Klenk.** 1996. Emerging and reemerging of filoviruses. Arch. Virol. **11**(Suppl.)**:**77–100.
13. **Feldmann, H., V. E. Volchkov, V. A. Volchkova, U. Stroher, and H. D. Klenk.** 2001. Biosynthesis and role of filoviral glycoproteins. J. Gen. Virol. **82:**2839–2848.
14. **Formenty, P., C. Hatz, B. Le Guenno, A. Stoll, P. Rogenmoser, and A. Widmer.** 1999. Human infection due to Ebola virus, subtype Cote d'Ivoire: clinical and biologic presentation. J. Infect. Dis. **179**(Suppl. 1)**:**S48–S53.
15. **Gear, J. S., G. A. Cassel, A. J. Gear, B. Trappler, L. Clausen, A. M. Meyers, M. C. Kew, T. H. Bothwell, R. Sher, G. B. Miller, J. Schneider, H. J. Koornhof, E. D. Gomperts, M. Isaacson, and J. H. Gear.** 1975. Outbreak of Marburg virus disease in Johannesburg. Br. Med. J. **iv:**489–493.
16. **Geisbert, T. W., and P. B. Jahrling.** 2004. Exotic emerging viral diseases: progress and challenges. Nat. Med. **10:**S110–S121.
17. **Georges-Courbot, M. C., A. Sanchez, C. Y. Lu, S. Baize, E. Leroy, J. Lansout-Soukate, C. Tevi-Benissan, A. J. Georges, S. G. Trappier, S. R. Zaki, R. Swanepoel, P. A. Leman, P. E. Rollin, C. J. Peters, S. T. Nichol, and T. G. Ksiazek.** 1997. Isolation and phylogenetic characterization of Ebola viruses causing different outbreaks in Gabon. Emerg. Infect. Dis. **3:**59–62.
18. **Gibb, T. R., D. A. Norwood, Jr., N. Woollen, and E. A. Henchal.** 2001. Development and evaluation of a fluorogenic 5′ nuclease assay to detect and differentiate between Ebola virus subtypes Zaire and Sudan. J. Clin. Microbiol. **39:**4125–4130.
19. **Groen, J., B. G. van den Hoogen, C. P. Burghoorn-Maas, A. R. Fooks, J. Burton, C. J. Clegg, H. Zeller, and A. D. Osterhaus.** 2003. Serological reactivity of baculovirus-expressed Ebola virus VP35 and nucleoproteins. Microbes Infect. **5:**379–385.
20. **Ikegami, T., M. Niikura, M. Saijo, M. E. Miranda, A. B. Calaor, M. Hernandez, L. P. Acosta, D. L. Manalo, I. Kurane, Y. Yoshikawa, and S. Morikawa.** 2003. Antigen capture enzyme-linked immunosorbent assay for specific detection of Reston Ebola virus nucleoprotein. Clin. Diagn. Lab. Immunol. **10:**552–557.
21. **Ikegami, T., M. Saijo, M. Niikura, M. E. Miranda, A. B. Calaor, M. Hernandez, D. L. Manalo, I. Kurane, Y. Yoshikawa, and S. Morikawa.** 2002. Development of an immunofluorescence method for the detection of antibodies to Ebola virus subtype Reston by the use of recombinant nucleoprotein-expressing HeLa cells. Microbiol. Immunol. **46:**633–638.
22. **Ikegami, T., M. Saijo, M. Niikura, M. E. Miranda, A. B. Calaor, M. Hernandez, D. L. Manalo, I. Kurane, Y. Yoshikawa, and S. Morikawa.** 2003. Immunoglobulin G enzyme-linked immunosorbent assay using truncated nucleoproteins of Reston Ebola virus. Epidemiol. Infect. **130:**533–539.
23. **Jahrling, P. B., T. W. Geisbert, D. W. Dalgard, E. D. Johnson, T. G. Ksiazek, W. C. Hall, and C. J. Peters.** 1990. Preliminary report: isolation of Ebola virus from monkeys imported to USA. Lancet **335:**502–505.
24. **Johnson, E. D., B. K. Johnson, D. Silverstein, P. Tukei, T. W. Geisbert, A. N. Sanchez, and P. B. Jahrling.** 1996. Characterization of a new Marburg virus isolated from a 1987 fatal case in Kenya. Arch. Virol. **11**(Suppl.)**:**101–114.
25. **Johnson, K. M., L. H. Elliott, and D. L. Heymann.** 1981. Preparation of polyvalent viral immunofluorescent intracellular antigens and use in human serosurveys. J. Clin. Microbiol. **14:**527–529.
26. **Johnson, K. M., J. V. Lange, P. A. Webb, and F. A. Murphy.** 1977. Isolation and partial characterisation of a new virus causing acute haemorrhagic fever in Zaire. Lancet **i:**569–571.
27. **Khan, A. S., F. K. Tshioko, D. L. Heymann, B. Le Guenno, P. Nabeth, B. Kerstiens, Y. Fleerackers, P. H. Kilmarx, G. R. Rodier, O. Nkuku, P. E. Rollin, A. Sanchez, S. R. Zaki, R. Swanepoel, O. Tomori, S. T. Nichol, C. J. Peters, J. J. Muyembe-Tamfum, and T. G. Ksiazek.** 1999. The reemergence of Ebola hemorrhagic fever, Democratic Republic of the Congo, 1995. Commission de Lutte contre les Epidemies a Kikwit. J. Infect. Dis. **179**(Suppl. 1)**:** S76–S86.
28. **Ksiazek, T. G., P. E. Rollin, P. B. Jahrling, E. Johnson, D. W. Dalgard, and C. J. Peters.** 1992. Enzyme immunosorbent assay for Ebola virus antigens in tissues of infected primates. J. Clin. Microbiol. **30:**947–950.
29. **Ksiazek, T. G., P. E. Rollin, A. J. Williams, D. S. Bressler, M. L. Martin, R. Swanepoel, F. J. Burt, P. A. Leman, A. S. Khan, A. K. Rowe, R. Mukunu, A. Sanchez, and C. J. Peters.** 1999. Clinical virology of Ebola hemorrhagic fever (EHF): virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. J. Infect. Dis. **179**(Suppl. 1)**:**S177–S187.
30. **Ksiazek, T. G., C. P. West, P. E. Rollin, P. B. Jahrling, and C. J. Peters.** 1999. ELISA for the detection of antibodies to Ebola viruses. J. Infect. Dis. **179**(Suppl. 1)**:**S192–S198.
31. **Le Guenno, B., P. Formenty, and C. Boesch.** 1999. Ebola virus outbreaks in the Ivory Coast and Liberia, 1994–1995. Curr. Top. Microbiol. Immunol. **235:**77–84.
32. **Leroy, E. M., S. Baize, C. Y. Lu, J. B. McCormick, A. J. Georges, M. C. Georges-Courbot, J. Lansoud-Soukate, and S. P. Fisher-Hoch.** 2000. Diagnosis of Ebola haemorrhagic fever by RT-PCR in an epidemic setting. J. Med. Virol. **60:**463–467.
33. **Leroy, E. M., B. Kumulungui, X. Pourrut, P. Rouquet, A. Hassanin, P. Yaba, A. Delicat, J. T. Paweska, J. P. Gonzalez, and R. Swanepoel.** 2005. Fruit bats as reservoirs of Ebola virus. Nature **438:**575–576.
34. **Lucht, A., R. Grunow, P. Moller, H. Feldmann, and S. Becker.** 2003. Development, characterization and use of monoclonal VP40-antibodies for the detection of Ebola virus. J. Virol. Methods **111:**21–28.
35. **Lucht, A., R. Grunow, C. Otterbein, P. Moller, H. Feldmann, and S. Becker.** 2004. Production of monoclonal antibodies and development of an antigen capture ELISA directed against the envelope glycoprotein GP of Ebola virus. Med. Microbiol. Immunol. (Berlin) **193:**181–187.
36. **Martini, G. A., H. G. Knauff, H. A. Schmidt, G. Mayer, and G. Baltzer.** 1968. On the hitherto unknown, in monkeys originating infectious disease: Marburg virus disease. Dtsch. Med. Wochenschr. **93:**559–571.
37. **Meissner, F., T. Maruyama, M. Frentsch, A. J. Hessell, L. L. Rodriguez, T. W. Geisbert, P. B. Jahrling, D. R. Burton, and P. W. Parren.** 2002. Detection of antibodies against the four subtypes of Ebola virus in sera from any species using a novel antibody-phage indicator assay. Virology **300:**236–243.
38. **Niedrig, M., H. Schmitz, S. Becker, S. Gunther, J. ter Meulen, H. Meyer, H. Ellerbrok, A. Nitsche, H. R. Gelderblom, and C. Drosten.** 2004. First international quality assurance study on the rapid detection of viral agents of bioterrorism. J. Clin. Microbiol. **42:**1753–1755.
39. **Niikura, M., T. Ikegami, M. Saijo, I. Kurane, M. E. Miranda, and S. Morikawa.** 2001. Detection of Ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. J. Clin. Microbiol. **39:**3267–3271.
40. **Niikura, M., T. Ikegami, M. Saijo, T. Kurata, I. Kurane, and S. Morikawa.** 2003. Analysis of linear B-cell epitopes of the nucleoprotein of Ebola virus that distinguish Ebola virus subtypes. Clin. Diagn. Lab. Immunol. **10:**83–87.
41. **Okome-Nkoumou, M., and M. Kombila.** 1999. A case of Ebola virus hemorrhagic fever in Libreville (Gabon), fatal after evacuation to South Africa. Med. Trop. (Mars) **59:**411.
42. **Peters, C. J., and J. W. LeDuc.** 1999. An introduction to Ebola: the virus and the disease. J. Infect. Dis. **179**(Suppl. 1)**:**ix–xvi.
43. **Prehaud, C., E. Hellebrand, D. Coudrier, V. E. Volchkov, V. A. Volchkova, H. Feldmann, B. Le Guenno, and M. Bouloy.** 1998. Recombinant Ebola virus nucleoprotein and glycoprotein (Gabon 94 strain) provide new tools for the detection of human infections. J. Gen. Virol. **79:**2565–2572.
44. **Saijo, M., M. Niikura, A. Maeda, T. Sata, T. Kurata, I. Kurane, and S. Morikawa.** 2005. Characterization of monoclonal antibodies to Marburg virus nucleoprotein (NP) that can be used for NP-capture enzyme-linked immunosorbent assay. J. Med. Virol. **76:**111–118.
45. **Saijo, M., M. Niikura, S. Morikawa, T. G. Ksiazek, R. F. Meyer, C. J. Peters, and I. Kurane.** 2001. Enzyme-linked immunosorbent assays for detection of antibodies to Ebola and Marburg viruses using recombinant nucleoproteins. J. Clin. Microbiol. **39:**1–7.
46. **Saijo, M., M. Niikura, S. Morikawa, and I. Kurane.** 2001. Immunofluorescence method for detection of Ebola virus immunoglobulin G, using HeLa cells which express recombinant nucleoprotein. J. Clin. Microbiol. **39:**776–778.
47. **Sanchez, A., A. S. Khan, S. Zaki, G. J. Nabel, T. G. Ksiazek, and C. J. Peters.** 2001. Filoviridae: Marburg and Ebola viruses, p. 1279–1304. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
48. **Sanchez, A., T. G. Ksiazek, P. E. Rollin, M. E. Miranda, S. G. Trappier, A. S. Khan, C. J. Peters, and S. T. Nichol.** 1999. Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. J. Infect. Dis. **179**(Suppl. 1)**:**S164–S169.
49. **Smith, D. H., B. K. Johnson, M. Isaacson, R. Swanapoel, K. M. Johnson, M. Killey, A. Bagshawe, T. Siongok, and W. K. Keruga.** 1982. Marburg-virus disease in Kenya. Lancet **i:**816–820.
50. **Towner, J. S., P. E. Rollin, D. G. Bausch, A. Sanchez, S. M. Crary, M. Vincent, W. F. Lee, C. F. Spiropoulou, T. G. Ksiazek, M. Lukwiya, F. Kaducu, R. Downing, and S. T. Nichol.** 2004. Rapid diagnosis of Ebola hemorrhagic fever by reverse transcription-PCR in an outbreak setting and assessment of patient viral load as a predictor of outcome. J. Virol. **78:**4330–4341.
51. **van der Groen, G., T. Kurata, and C. Mets.** 1983. Modifications to indirect immunofluorescence tests on Lassa, Marburg, and Ebola material. Lancet **i:**654.

52. **Volchkov, V., V. Volchkova, C. Eckel, H. D. Klenk, M. Bouloy, B. LeGuenno, and H. Feldmann.** 1997. Emergence of subtype Zaire Ebola virus in Gabon. Virology **232:**139–144.
53. **Weidmann, M., E. Muhlberger, and F. T. Hufert.** 2004. Rapid detection protocol for filoviruses. J. Clin. Virol. **30:**94–99.
54. **World Health Organization.** 1978. Ebola haemorrhagic fever in Sudan, 1976. Report of a WHO/International Study Team. Bull. W. H. O. **56:**247–270.
55. **World Health Organization.** 1978. Ebola haemorrhagic fever in Zaire, 1976. Bull. W. H. O. **56:**271–293.
56. **World Health Organization.** 2005. Marburg haemorrhagic fever, Angola. Wkly. Epidemiol. Rec. **80:**158–159.
57. **World Health Organization.** 1996. Outbreak of Ebola haemorrhagic fever in Gabon. Commun. Dis. Rep. CDR Wkly. **6:**75, 78.
58. **World Health Organization.** 2001. Outbreak of Ebola haemorrhagic fever, Uganda, August 2000-January 2001. Wkly. Epidemiol. Rec. **76:**41–46.
59. **World Health Organization.** 1995. Outbreak of Ebola hemorrhagic fever—Zaire, 1995. Epidemiol. Bull. **16:**16.
60. **World Health Organization.** 2005. Outbreak of Marburg virus hemorrhagic fever—Angola, October 1, 2004-March 29, 2005. Morb. Mortal. Wkly. Rep. **54:**308–309.
61. **World Health Organization.** 2003. Outbreak(s) of Ebola haemorrhagic fever in the Republic of the Congo, January-April 2003. Wkly. Epidemiol. Rec. **78:**285–289.
62. **World Health Organization.** 2003. Outbreak(s) of Ebola hemorrhagic fever, Congo and Gabon, October 2001 to July 2002. Can. Commun. Dis. Rep. **29:**129–133.
63. **World Health Organization.** 1992. Viral haemorrhagic fever in imported monkeys. Wkly. Epidemiol. Rec. **67:**142–143.
64. **Zaki, S. R., W. J. Shieh, P. W. Greer, C. S. Goldsmith, T. Ferebee, J. Katshitshi, F. K. Tshioko, M. A. Bwaka, R. Swanepoel, P. Calain, A. S. Khan, E. Lloyd, P. E. Rollin, T. G. Ksiazek, and C. J. Peters.** 1999. A novel immunohistochemical assay for the detection of Ebola virus in skin: implications for diagnosis, spread, and surveillance of Ebola hemorrhagic fever. Commission de Lutte contre les Epidemies a Kikwit. J. Infect. Dis. **179** (Suppl. 1)**:**S36–S47.